PATENT
CUSTOMER No. 32425

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Johan FROSTEGARD	Group Art Unit: 1641
Serial No.: 10/814,125	Examiner: LISA V. COOK
Filed: April 1, 2004	Atty. Dkt. No.: EPCL:010US
For: METHOD OF DIAGNOSING CARDIOVASCULAR DISEASE	Confirmation No.: 8029

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Steven L. Highlander

REPLY BRIEF

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REPLY BRIEF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-01450

Dear Sir:

This Reply Brief is filed in response to the Examiner's Answer mailed on June 16, 2008. Appellants' reply is due August 16, 2008. Also accompanying this filing is a Request for Oral Argument and payment of fees. No other fees are believed due in connection with this filing; however, should appellants payments be missing or deficient, or should any fees be due, appellants authorize the Commissioner to debit Fulbright & Jaworski L.L.P. Deposit Account No. 50-1212/EPCL:010US/SLH.

I. Real Party In Interest

The real party in interest is the assignee, Athera Biotechnologies, AB, Stockholm, Sweden.

II. Related Appeals and Interferences

There are no related appeals or interferences.

III. Status of the Claims

A copy of the appealed claims is attached as Appendix A.

IV. Status of the Amendments

No "after final" amendments have been presented.

V. Summary of the Claimed Subject Matter

Claim 1, drawn to a method for diagnosing early cardiovascular disease, is supported in the specification as follows: (a) contacting a sample of body fluid with phosphocholine and/or a derivative thereof (Specification at paras. [0013-0014, 0016]), (b) assessing the presence and/or concentration of antibodies to phosphocholine and/or to said derivative in the sample by measuring antibodies bound to phosphocholine and/or derivative thereof (Specification at para. [0014]), and (c) diagnosing early cardiovascular disease based on the presence and/or concentration of said antibodies in the sample (Specification at para. [0004, 0013]).

VI. Remaining Grounds of Rejection to be Reviewed on Appeal

1. Claims 1-3, 6-8, 11-14, 16, 17, 20-23 and 26 as allegedly obvious over Muzya *et al.* (Exhibit 2) in view of Ostermann *et al.* (Exhibit 4).
2. Claims 4, 9, 18 and 24 as allegedly obvious over Muzya *et al.* (Exhibit 2) in view of Ostermann *et al.* (Exhibit 4) and Barquinero *et al.* (Exhibit 5).
3. Claims 5, 10, 19 and 25 as allegedly obvious over Muzya *et al.* (Exhibit 2) in view of Ostermann *et al.* (Exhibit 4) and Smal *et al.* (Exhibit 6).

VII. Argument

A. Standard of Review

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. §706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312. Accordingly, it necessarily follows that an examiner's position on appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Rejections Under 35 U.S.C. §103

At the outset, appellants wish to draw the Board's attention to continued points of confusion on the part of the examiner which appellants have repeatedly attempted to clarify. In particular, the examiner is repeatedly confused by the differences between (1) platelet aggregating factor ("PAF"), which is the chemical 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (see paragraph [0025] of the application as filed); (2) antibodies which ***bind*** to PAF (also known as anti-PAF antibodies, or further abbreviated to "aPAF" – see paragraph [0039] of the application as filed); (3) and the enzyme PAF acetylhydrolase, which ***degrades*** PAF (see Ostermann, page 530, 2nd paragraph). The examiner has repeatedly confused the differences between these physically distinct molecules, and often also uses these terms interchangeably as though they mean the same thing. They do ***not*** mean the same thing,

however, and at the risk of belaboring the point, they refer to ***physically distinct molecules***. Thus, a teaching in the prior art of the measurement of the level of ***one*** such molecule ***cannot*** be equated with the measurement of the level of the ***other*** molecules.

(i) Claims 1-3, 6-8, 11-14, 16, 17, 20-23 and 26 as allegedly obvious over Muzya *et al.* in view of Ostermann *et al.*

In the Examiner's Answer at page 5, the examiner states that "Muzya *et al.* teach the reagents and methods required by the claims." This is not true. The appealed claims are directed to measuring levels of antibodies to phosphocholine ("PC") and/or derivatives thereof. However, Muzya's use of the term "PC" refers to phosphatidylcholine (for example, see the first sentence of the "Results and Discussion" section of Muzya), which is different from the molecule phosphocholine, as recited in the claims of the present application. It is important that the different uses of the abbreviation "PC" in these two documents are not confused.

Next, the examiner states that Muzya only differs from the present invention in not specifically teaching PAF as an indicator of CVD such as atherosclerosis (Answer, page 5). The examiner again incorrect, and this is one of the repeated examples of the examiner incorrectly using the terms "PAF" and "aPAF" interchangeably as if they mean the same thing. Contrary to the examiner's statement, Muzya teaches the assessment of levels of ***aPAF***, not PAF itself. The present invention relates to measuring levels of antibodies to PC and its derivatives (*i.e.*, aPC levels) not, as suggested by the examiner, the measurement of levels of PAF itself. Regardless, the examiner is correct in recognizing that Muzya relates to the assessment of a different medical condition than the subject matter of the claims.

Turning to Ostermann, at page 6 of the Answer, first full paragraph, the examiner states that this references teaches PAF quantification in serum and plasma and its correlation/diagnosis

in atherosclerotic patients. The examiner is again wrong. Ostermann teaches quantification of ***levels of the enzyme PAF acetylhydrolase***, which degrades PAF, not quantification of PAF as suggested by the examiner. It also does not teach either the quantification of any antibody levels or quantification of specific levels of antibodies to PC and/or its derivatives. This is further example of the examiner incorrectly using the terms PAF and PAF acetylhydrolase interchangeably. Moving further down on page 6, in the third paragraph makes the same misstatements about both Ostermann and Muzya ("measure PAF concentrations in serum and plasma .... teach that PAF could discriminate between low and high risk groups ....)

Moving forward to the examiner's response to appellants brief, at page 9, the examiner states that Ostermann is relied upon to establish the relationship between PAF-aPAF complexes measurements and early CVD. The examiner is again incorrect. Ostermann relates to the levels of the enzyme ***PAF acetylhydrolase*** (which degrades PAF), not quantification of PAF or of any antibody levels, much less specifically the levels of antibodies to PC and/or its derivatives.

Further at page 9, the examiner challenges appellants' contention that the claims are directed to measuring levels of antibodies to phosphocholine ("PC") and/or derivatives thereof, arguing that "the claims are drawn not only to the concentration of the antibody bound to PAF but the presence of said antibodies bound to PAF." Once again, the examiner is hopelessly confused. The examiner seems to be suggesting that the claims are drawn to an assay that detects the abundance of PAF-aPAF complexes (*i.e.*, complexes between ligand and antibody) in the serum of a patient and (perhaps by extrapolation) gives an indication of PAF levels in the patient. This is wholly incorrect. The *in vivo* level of such complexes, or of PAF itself, is not assayed. Rather, the claims are drawn to an assay for ***levels of antibodies*** that have the ability to bind to PC and/or its derivatives, which is assayed *in vitro* by detecting antibody binding to

artificially-provided PC or its derivatives (*e.g.*, where PC is coated onto the surface of a well in an ELISA assay). There is no indication that the level of antibody-PAF complexes existing *in vivo* is assayed.

At page 10, first paragraph, the examiner seems to suggest, that Ostermann teaches the measurement of interaction between PAF and antibodies present in a sample. This is totally incorrect. Ostermann teaches the interaction between PAF and a PAF degrading enzyme (not an antibody), wherein PAF is added artificially to the sample to assay the level of enzyme present in the sample. There is no disclosure in Ostermann of measuring PAF levels, and certainly not aPAF levels.

Further on in page 10, the examiner states that "Ostermann was combined with Muzya to make obvious the relationship between early cardiovascular disease and the complex formed between PAF and antibodies to PAF." Yet Ostermann says nothing about the relationship between early CVD and the formation of a complex between PAF and antibodies to PAF. As has been repeatedly pointed out, Ostermann is ***only*** concerned with serum levels of ***a PAF-degrading enzyme***. It is certainly not concerned with levels of aPC as specified by the appealed claims.

Next, the examiner states that she has not relied on Ostermann for the teaching of PAF levels or anti-PAF antibody levels in subjects. This is in ***direct contradiction*** to the examiner's statement on page 9, where she stated that "The reference to Ostermann was merely employed to establish the relationship between PAF/antibodies to PAF complex measurements and early cardiovascular disease." Thus, the examiner cannot even state clearly the propositions for which the references are or are not being cited.

At the bottom of page 10, the examiner states that "In addition, Ostermann teaches a method of adding ^{14}C-PAF ... to serum samples ... and measuring the interaction of PAF bound to antibodies to PAF. Once again, the examiner misunderstands the teachings of Ostermann. Ostermann does not teach measuring the interaction of PAF with antibodies to PAF, but teaches use of radiolabelled PAF to assay the level of ***a PAF-degrading enzyme*** in the serum of a patient.

The examiner does correctly note, at the top of page 11, that Ostermann is concerned with assessing the PAF-degrading capacity of serum, but then fails to admit that the capacity of serum to effect PAF-degradation (which is a measurement of the serum level of the responsible enzyme, PAF acetylhydrolase) is not in any way correlated with the serum level of PAF, nor with the serum level of PAF binding with the antibody aPAF, nor the serum level of aPAF itself.

Further on at page 11, the examiner indicates that Ostermann shows that PAF plays a role in the development of atherosclerosis, but again, Ostermann's data only relates to the role of the enzyme PAF acetylhydrolase. This is not PAF, it is certainly not an antibody to PAF, and more specifically it is not aPC as recited by the claims. The examiner attempts to avoid this by arguing that "regardless of the method of measurement taught by Ostermann the relationship between early cardiovascular disease and PAF/antibodies bound to PAF is clearly identified." This statement could not be more incorrect. First, it ***does*** matter that Ostermann looked at PAF-degrading enzyme levels, and not PAF. There is not one shred of evidence that there is a correlation in CVD patients between PAF acetylhydrolase and PAF levels. Second, there is nothing in the claims regarding PAF-aPAF complexes, and thus this comment is completely and utterly irrelevant.

In sum, appellants submit again that the cited references fail to render obvious the claimed invention for reasons already made of record – that Muzya deals with gynecologic orders, not CVD, as well as phosphatidylcholine, not phosphocholine, while Ostermann deals with PAF acetylhydrolase levels, and not PAF levels. Moreover, the failure of the examiner to correctly cite to the teachings of these references renders the entire rejection non-sensical and hence improper. For these reasons, reversal of the rejection is again respectfully requested.

(ii) Claims 4, 9, 18 and 24 as allegedly obvious over Muzya *et al.* in view of Ostermann *et al.* and Barquinero *et al.*

At page 7 of the action, the examiner adds Barquinero to the citations of Muzya and Ostermann, merely for the teaching of ELISA formats for aPAF. This cannot correct the numerous deficiencies of Muzya and Ostermann, and the examiner's supporting argumentation, as discussed above and in previous submissions. Thus, this rejection is improper and should be reversed as well.

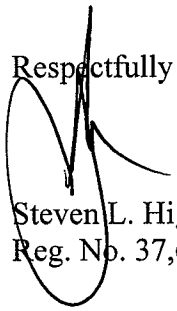
(iii) Claims 5, 10 19 and 25 as allegedly obvious over Muzya *et al.* in view of Ostermann *et al.* and Smal *et al.*

At page 9 of the action, the examiner adds Smal to the teachings of Muzya and Ostermann, merely for the teaching of radioimmunoassays. This cannot correct the numerous deficiencies of Muzya and Ostermann, and the examiner's supporting argumentation, as discussed above and in previous submissions. Thus, this rejection is improper and should be reversed as well.

C. Conclusion

In light of the foregoing, appellants respectfully submit that all pending claims are enabled and non-obvious over the cited art. Therefore, it is respectfully requested that the Board reverse each of the pending rejections.

Respectfully submitted,



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Date: August 15, 2008

VIII. APPENDIX A – APPEALED CLAIMS

1. A method for diagnosing early cardiovascular disease comprising (a) contacting a sample of body fluid with phosphocholine and/or a derivative thereof, (b) assessing the presence and/or concentration of antibodies to phosphocholine and/or to said derivative in the sample by measuring antibodies bound to phosphocholine and/or derivative thereof, and (c) diagnosing early cardiovascular disease based on the presence and/or concentration of said antibodies in the sample.

2. The method of claim 1, wherein said early cardiovascular disease comprises atherosclerosis, hypertension or thrombosis.

3. The method of claim 2, wherein measuring comprises an immunoassay.

4. The method of claim 2, wherein measuring comprises an enzyme linked immunosorbent assay.

5. The method of claim 2, wherein measuring comprises a radioimmunoassay.

6. The method of claim 2, wherein said body fluid is serum prepared from a blood sample.

7. The method of claim 2, wherein said body fluid is plasma prepared from a blood sample.

8. The method of claim 1, wherein measuring comprises an immunoassay.

9. The method of claim 1, wherein measuring comprises an enzyme linked immunosorbent assay.

10. The method of claim 1, wherein measuring comprises a radioimmunoassay.

11. The method of claim 1, wherein said body fluid is serum prepared from a blood sample.

12. The method of claim 1, wherein said body fluid is plasma prepared from a blood sample.

13. The method of claim 1, wherein said body fluid is a human blood sample or fraction thereof, and said measuring comprises an immunoassay.

14. The method of claim 2, wherein said derivative is lysophosphatidylcholine.

16. The method of claim 1, wherein said derivative is lysophosphatidylcholine.

17. The method of claim 3, wherein said derivative is lysophosphatidylcholine.

18. The method of claim 4, wherein said derivative is lysophosphatidylcholine.

19. The method of claim 5, wherein said derivative is lysophosphatidylcholine.

20. The method of claim 6, wherein said derivative is lysophosphatidylcholine.

21. The method of claim 1, wherein said body fluid is contacted with phosphochline.

22. The method of claim 2, wherein said body fluid is contacted with phosphochline.

23. The method of claim 3, wherein said body fluid is contacted with phosphochline.

24. The method of claim 4, wherein said body fluid is contacted with phosphochline.

25. The method of claim 5, wherein said body fluid is contacted with phosphochline.

26. The method of claim 6, wherein said body fluid is contacted with phosphochline.

EXHIBIT 2

UDC 618.3-092:812.087.1]-078-33

Reaction of antiphosphatidylcholine antibodies with thrombocyte-activating phospholipid factor and its structural cellular analogues

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The high proportion of antiphospholipid antibodies to membrane phospholipids is often associated with obstetrical pathology; this includes recurrent foetal loss, intrauterine growth retardation, hypertension in pregnancy, preeclampsia and thromboembolic complications [15]. In such cases, the blood serum of patients contains antibodies to the main cellular phospholipids such as cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, sphyngomyelin and phosphatidylcholine [10]. Study of the specific mechanisms of antiphospholipid antibody participation in the development of the pathology of pregnancy continues [14, 15]. It has been suggested that the rise observed in pregnancy in the level of phospholipids in the blood due to an increase in their anabolism, the deportation of syncytiotrophoblast microvilli and the release of phospholipid vesicles by the placenta stimulate the production of antiphospholipid antibodies [10].

Antiphospholipid antibodies (aPL) are apparently reactive not only with 'excess' phospholipids in the blood serum but also with lipoproteins and cells containing phospholipid antigen determinants on the cell surface [14].

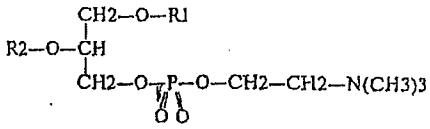
It is known that given the correct stimulus virtually all cells in mammals will release a universal phospholipid bioregulator, platelet-activating factor (PAF) [3] and its choline-containing cellular analogues - acyl and plasmalogen [4]. Phospholipid PAF is involved in the regulation of the blood clotting system, the cardio-vascular system and the immune system and is a mediator of inflammation with a range of etiologies, of allergic reactions and many other pathophysiological processes. PAF plays a major role in mammalian reproduction, with an effect on virtually all stages of the reproductive process, from functional development of the gametes, fertilisation and

embryo implantation to childbirth [6]. Since PAF is in terms of its chemical structure a choline-containing phospholipid, it may be expected that aPL antibodies and aPC antibodies in particular would be reactive with PAF and its structural analogues, with an effect on their biological activity.

The aim of this study was to investigate the reaction of blood serum containing antiphosphatidylcholine antibodies with PAF and its structural analogues.

Research method

Preparations of highly purified phospholipids of the following structure were used in the research project.



1) phosphatidylcholine: R1 is C16:0 and C18:0 fatty acid residues; R2 is C18:1 and C18:2 fatty acid residues
2) lysophosphatidylcholine: R1 is C16:0 and C18.0 fatty acid residues; R2 is H
3) phospholipid PAF: R1 is (CH2)15,17CH3; R2 is CH3CO;
4) PAF lysoderivative (lyso-PAF): R1 is (CH2)15, 17CH3; R2 is H
5) acyl analogue of PAF (1-acyl-PAF): R1 is C16:0 and C18.0 fatty acid residues; R2 is CH3CO.

Phosphatidylcholine is separated from egg yolks by the usual method [1]. Lysophosphatidylcholine (1-acyllysoglycero-3-phosphocholine) was obtained by cleaving egg phosphatidylcholine with phospholipase A2 and purified by column chromatography in L 100/160 μm silica gel [1]. 1-0-alkyllyso-*sn*-glycero-3-phosphocholine (lyso-PAF) was obtained by hydrogenating bovine heart choline plasmalogens, followed by alkaline hydrolysis as described earlier [1]. Phospholipid PAF was obtained by acetylating 1-0-alkyllyso-sn-glycero-3-phosphocholine with acetic anhydride in a chloroform medium and purifying by column chromatography in L 100/160 μm silica gel [2]. The PAF acyl analogue (1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine) was obtained by acetylating the lysophophatidylcholine with acetic anhydride in a chloroform medium and purifying by column chromatography in L 100/160 μm silica gel [7].

Murine monoclonal antibodies to human immunoglobulins (IgM, IgG), labelled horseradish peroxidase (Institute of Virus Preparations, Moscow), gelatine (N.A. Semashko Moskhimpharmpreparat), o-phenylendiamine (Sigma), hydrogen peroxide (Reakhim) and polystyrene microplates manufactured by GosNIIMedpolimer (Moscow) were used for the enzyme immunoassay (EIA). Blood serum samples taken in the Scientific Centre of Obstetrics, Gynaecology and perinatology of the Russian Academy of Medical Sciences from patients with recurrent foetal loss, late toxicosis in pregnancy, history of perinatal foetal death, infertility and unsuccessful attempts at *in vitro* fertilisation and embryo transfer.

EIA was used to study the manner in which aPL antibodies bind with PAF and its structural analogues. Highly purified phospholipids (phosphatidylcholine, PAF, lyso-PAF, 1-acyl-PAF, lysophosphatidylcholine) were dissolved in a 50 μg/ml methanol concentration. The resultant phospholipid solutions were placed onto polystyrene microplates in quantities of 50 μm per well and incubated at 37°C for 18 ± 2 hrs. After each stage of the assay the plates were washed 4 times with 0.01 M phosphate buffer solution (pH 7.4 ± 0.2). After adsorption of the phospholipids the wells were treated with a 0.5% gelatine solution, 100 μm per well, at 20 ± 2 °C for 1.5 hr. A phosphate buffer solution containing 0.5% gelatine was used for cultivating the test samples of blood serum and conjugates. 75 μl assay samples of blood serum cultivated in a 1:50 proportion were inserted per well and incubated at 20 ± 2 °C in an agitator for 1.5 hr. Conjugates of murine monoclonal antibodies, with horseradish peroxidase, to human IgM and IgG, in 1:100000 and 1:50000 proportions respectively, were placed in the wells in amounts of 50 μl per well and incubated at 20 ± 2 °C in an agitator for 1 hr. After washing, a chromogen substrate solution containing o-phenylendiamine and hydrogen peroxide was added to the wells and the optical density (OD) was measured after 10 minutes at 492 nm using a Labsystems Multiscan MCC/340 photometer. The results of the assay were considered positive if the average OD of the assay sample was greater than the total of the average OD for the negative controls and two average mean square deviations.

Results and Discussion

To study the way antiphosphatidylcholine (aPC) antibodies bind with phospholipid PAF and its structural analogues, blood serum containing IgM, or IgM and IgG phosphatidylcholine antibodies was taken from patients presenting with obstetric and gynaecological pathologies. In

the case of the patient with late toxicity in pregnancy the IgG level was relatively higher than the IgM level, while in the other cases the IgM level was higher.

The EIA results indicated that serums containing IgM and IgG aPC antibodies react *in vitro* with the PAF and its analogues adsorbed onto the polystyrene plates. In addition, the linking of IgM antibodies with phosphatidylcholine was approximately 1.5 - 2 times higher than with PAF, lyso-PAF and 1-acyl-PAF adsorbed under the same conditions, and 3 times higher than with lysophosphatidylcholine (Table 1). No substantial differences were found in the degree of the reaction of aPC antibodies with PAF, lysoPAF and 1-acyl-PAF. The cross reaction typical for antiphospholipid antibodies had obviously occurred in this case.

Table 1

Level of IgM aPC antibodies in blood serum of patients with obstetric and gynaecological pathologies, and the binding with PAF and its structural analogues found from EIA

Patient group	Phospholipid tested				
	Phosphatidyl-choline	PAF	Lyso-PAF	1-acyl-PAF	Lyso-phosphatidyl-choline
Patients with death of infant in neonatal period	0.580 ± 0.035	0.759 ± 0.044	0.387 ± 0.023	0.386 ± 0.022	0.268 ± 0.015
Patients with foetal loss	0.320 ± 0.016	0.576 ± 0.034	0.243 ± 0.014	0.208 ± 0.012	0.145 ± 0.018
Patients with late toxicosis in pregnancy	0.400 ± 0.024	0.645 ± 0.065	0.293 ± 0.017	0.229 ± 0.013	0.378 ± 0.022
Patients with infertility	0.541 ± 0.031	0.727 ± 0.073	0.410 ± 0.024	0.356 ± 0.021	0.268 ± 0.016
Healthy fertile women	0.050 ± 0.003	0.126 ± 0.007	0.062 ± 0.004	0.051 ± 0.003	0.074 ± 0.004

Note: In Tables 1 and 2 the values given are for average OD at 492 nm ± σ

In the serum of patients with low levels of IgG aPC antibodies the differences in the way they bind with PAF and its analogues were slight (Table 2). However in the serum of the patient with late toxicosis in pregnancy a high level of IgG antibodies reactive with PAF and, significantly, to a lesser extent with its analogues was noted. It is not impossible that this patient had specific antibodies to PAF.

Table 2

Level of IgG aPC antibodies in blood serum of patients with obstetric and gynaecological pathologies, and the binding with PAF and its structural analogues found from EIA

Patient group	Phospholipid tested				
	Phosphatidyl-choline	PAF	Lyso-PAF	1-acyl-PAF	Lyso-phosphatidyl-choline
Patients with death of infant in neonatal period	0.189 ± 0.011	0.227 ± 0.013	0.141 ± 0.015	0.098 ± 0.006	0.086 ± 0.005
Patients with foetal loss	0.126 ± 0.008	0.250 ± 0.016	0.127 ± 0.009	0.089 ± 0.006	0.076 ± 0.008
Patients with late toxicosis in pregnancy	0.574 ± 0.034	1.011 ± 0.059	0.152 ± 0.010	0.097 ± 0.006	0.149 ± 0.009
Patients with infertility	0.085 ± 0.005	0.221 ± 0.014	0.114 ± 0.007	0.089 ± 0.006	0.050 ± 0.003
Healthy fertile women	0.061 ± 0.004	0.134 ± 0.008	0.064 ± 0.004	0.057 ± 0.004	0.050 ± 0.003

It is known that the antibodies to PAF may be evoked in rabbits after the introduction of PAF preparations containing C6:0 and C:12 alkyl residues, and PAF analogues (1-0-(ω-oxyalkyl)-2-acetyl-*sn*-glycero-3-phosphocholine, 1-0-(15'-carboxypentadecyl)-2-N, N-dimethylcarbamoyl-*sn*-glycero-3-phosphocholine), covalently linked to methylated BSA [8, 11, 17, 18]. The identified antibodies to PAF were highly specific and were not reactive with lyso-PAF, PAF enantiomer, PAF methoxy analogue, lysophosphatidylcholine, phosphatidylcholine or PAF analogues containing propionic or butyric acid residues at the *sn*-2 position [8, 11, 17]. With the different molecular types of PAF containing C16:0, C18:0 and C18:1 alkyl residues at the *sn*-1 position, there were some small variations in the bonding of the antibodies, and the greatest bonding of antibodies was observed in C18:1 PAF [8]. These results indicate that the high specificity of antibodies to PAF depends on the recognition of the acetyl group at the *sn*-2 position and the trimethylammonium group of phosphocholine in the PAF molecule [17].

In contrast to the highly specific antibodies to PAF, aPC antibodies are not highly specific and are reactive with other phospholipids. It has been shown that antibodies to phosphatidcholine can be evoked in experimental animals by introducing erythrocytes, an emulsion of dipalmitoyl phosphatidylcholine in BSA or phosphatodylcholine liposomes, and they can also be produced by hybridoma technology [9, 12, 13, 16, 19]. aPC antibodies are also capable of binding with

lyso-phosphatidylcholine and sphyngomyelin [12], that is, they are capable of recognition of phosphocholine fragments of the polar part of phospholipids.

The results of this study show that IgM and IgG aPC antibodies in blood serum from patients with obstetric and gynaecological pathologies are capable of binding *in vitro* with PAF and its structural analogues which differ from PAF in the type of bond at the *sn*-1 position: a simple ether bond in the case of PAF and an ester bond in the case of 1-acyl-PAF.

What are implications of this observable reaction of aPC antibodies with PAF and its analogues in the pathogenesis of antiphospholipid syndrome (APS)? Thrombosis of the vessels of the placenta is thought to be the main mechanism in the development of obstetric pathology, with one of the causes of its occurrence being the major role played by the reaction of aPL antibodies with endothelial cells and thrombocytes [14]. It has been shown that the binding of aPL antibodies with endothelial cells leads to a reduction in the synthesis of prostacyclin, while their reaction with thrombocytes initiates the activation of thrombocytes and subsequent increase in the synthesis of thromboxane A2 and the release of adenosine diphosphate (ADP) [15]. At the same time, due to the presence of anticardiolipin antibodies, the endothelial cells release PAF [6]. Thus the increased production by cells of proaggregating agents such as PAF, thromboxane A2 and ADP along with the reduction in the synthesis of prostacyclin can cause the formation of intravascular aggregates of thrombocytes.

It is known that, in the blood circulation, PAF, released by cells binds with albumin and plasma lipoproteins [2] while free PAF is cleaved by acetylhydrolase associated with low density lipoproteins [5]. aPC antibodies can, apparently, bind with PAF in the microenvironment of cells actively producing PAF. It can be suggested that the formation of a compound with an antibody can inhibit the cleavage of PAF by acetylhydrolase.

An another important implication of the reaction of aPC antibodies with PAF may be the disturbance of the process of fertilisation of oocytes by spermatozoids. It is known that PAF stimulates spermatozoid motility, the acrosome reaction, and the process of fertilisation and implantation of the embryo [6]. Apparently aPC antibodies can significantly disturb these processes by removing PAF from the interaction of cells in the reproductive system. It is possible that unsuccessful attempts at *in vitro* fertilisation may be associated with a disturbance of the process of fertilisation and implantation of the embryo as a result of the binding of PAF

with aPC antibodies. It is therefore possible to suggest new links between APS and disturbances of the fertilisation processes in humans.

CONCLUSIONS

1. Antiphosphatidylcholine antibodies in the blood serum of patients with an obstetric and gynaecological pathology bind *in vitro* with phospholipid PAF, PAF lysine derivatives and PAF acyl analogues.

2. Antiphosphatidylcholine antibodies bound with PAF and its structural cell analogues are likely to be associated with the presence of phosphocholine fragments in the structure of certain phosphoglycerides.

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Trans notes:
1. *thrombocyte activating phospholipid factor*: The literal translation has been used in the title, since it is a title. Elsewhere in the text the more usual English platelet activating factor, PAF, has been used.

2. *structural cellular analogues*: this Russian term has been shortened to 'structural analogues' throughout the translation.

3. *Patient*: in this text, the Russian uses the word 'female patient'.

4. *Phosphatidylinositol*: This term has been used to translate the Russian 'phosphatidylinosite'.

5. *late toxicosis in pregnancy*: the Russian term, 'OPG-gestoz', was introduced in 1987 for late toxicosis in pregnant women; the *O G P* stands for oedema, proteinuria and hypertension.

EXHIBIT 4

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THE DEGRADATION OF PLATELET-ACTIVATING FACTOR IN SERUM AND ITS DISCRIMINATIVE VALUE IN ATHEROSCLEROTIC PATIENTS

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ABSTRACT

Platelet-activating factor (PAF) is transformed in vivo rapidly into the biologically inactive lyso-PAF. This reaction as well as lipid parameters were quantified in serum from 40 survivors of myocardial infarction and 36 healthy controls matched for age and body weight. The PAF-degrading capacity was 23% ($p<0.001$) higher in patients compared with the control group. Using the degradation of PAF as an univariate discriminator more than 70% of subjects were classified correctly. This is comparable with the discriminatory value of the best lipid variables, apolipoprotein B and HDL-cholesterol. Statistically significant differences in the degradation of PAF were found also by comparing subgroups which were matched for plasma levels of total cholesterol, VLDL/LDL-cholesterol or apolipoprotein B. The ratio HDL-cholesterol/degradation of PAF which is increased by 48 % ($p<0.0001$) in the case group was identified as an additional good discriminator between both groups. In contrast, platelet aggregation tests which were performed in acetylsalicyclic acid treated platelet-rich plasma discriminated poorly between patients and controls.

Key words: Platelet-Activating Factor, Lipoproteins, Platelet Function Tests, Atherosclerosis

INTRODUCTION

Platelet-activating factor (PAF, 1-O-alkyl-2-O-acetyl-sn-glycero-3-phosphocholine) is an extremely potent lipid mediator [1] which is considered to be involved in various inflammatory, respiratory, and cardiovascular disorders [2].

The effects of PAF are limited *in vivo* by a rapid removal of the 2-O-acetyl group [3]. This reaction is catalyzed by a PAF-specific acetylhydrolase [4] which is found in various tissues and cells as well as in serum or plasma. The plasmic PAF-acetylhydrolase which has been purified recently [5] is strongly bound to lipoproteins [6-8] and has properties somewhat different from those of the cellular enzyme [9]. Investigations on the uptake and degradation of PAF by individual lipoproteins revealed that the lipoproteins play a more complex role in the degradation of PAF than simply binding PAF-acetylhydrolase [10]. Moreover, we recently have shown, that the degradation of PAF in serum and plasma correlates highly significant with the lipoprotein profile [7] and is increased in patients suffering from peripheral vascular disease [11]. Abnormal high PAF-acetylhydrolase activities were found also in plasma of patients with familial LCAT deficiency [12] and a case of Tangier disease [13] which is characterized by the virtual absence of high density lipoproteins.

In view of these manifold relationships between the lipoprotein profile and the degradation of PAF and the well known role of lipoproteins as important risk factor for atherosclerosis [14], the present study was undertaken to establish whether serum PAF-acetylhydrolase is useful to discriminate between patients suffering from cardiovascular diseases and healthy subjects.

For this purpose the degradation of PAF and various lipid variables were measured in serum from survivors of myocardial infarction and an age- and body weight matched control group. Additionally, some functional parameters of platelets representing one of the target cells of PAF which play also an important role in atherogenesis [15], were included in this study.

MATERIALS AND METHODS

Chemicals and reagents: ^{14}C-PAF was prepared by reacting 1-O-hexadecyl-rac-glycero-3-phosphocholine with ^{14}C-acetic anhydride (30.5 MBq/mg, Isocommerz GmbH, Berlin, GDR) in anhydrous pyridine as described previously [16]. The labelled compound was chromatographed on silica gel resulting in a radiopurity of greater than 95%. It was dissolved in albumin-PBS (2.5 mg human serum albumin per ml of phosphate-buffered saline, pH 7.4), stored at -20°C, and further diluted with albumin-PBS immediately before use.

Subjects: Two groups of male subjects, 40 patients with clinical evidence of atherosclerotic diseases and 36 age- and body weight-matched healthy subjects were included into this study. The case group was recruited from outpatients of the

Clinic of Internal Medicine of the Medical Academy of Erfurt and had survived a myocardial infarction at least one year before their entry into this study. Myocardial infarction was documented by specific criteria including electro cardiographic changes, elevated serum enzymes, and typical symptoms. The control group consisted of healthy volunteers who had no known history of symptoms of heart disease [17].

Blood sampling und platelet preparation: Blood was always taken by venipuncture after an overnight fasting. Serum and heparin plasma were separated by centrifugation and stored frozen until further analysis. To prepare platelets, blood was collected into 0.1 volume citrate/ASA (0.11 mol/l trisodiumcitrate / 0.5 mmol/l acetylsalicylic acid) and centrifuged for 10 min at 200xg. The platelet-rich plasma (PRP) was aspirated, adjusted to $2.5\pm0.2\times10^8$ platelets/ml with autologous platelet-poor plasma (PPP), and stored in tightly stoppered plastic tubes at room temperature. PPP was obtained by centrifugation of PRP for 10 min at 1400xg. Aggregation tests were started 90 min after blood collection.

PAF-degrading capacity: The degradation of PAF in serum was measured under standard conditions by a method similar to that described by BLANK et al. [4]. 50 µl serum dilution (1:19) were added to 0.5 ml of 11 µM ^{14}C-PAF at a temperature of 37°C. After 5 and 10 min the reaction was terminated by transferring aliquots of 0.2 ml into 0.6 ml of ice-cold chloroform/methanol (2/1, v/v). The samples were mixed and then centrifuged at 8000xg for 3 min. The upper phase was removed, washed with chloroform and finally the amount of ^{14}C-acetate was assayed by liquid szintillation counting. Mean values of four separate incubations were used.

Lipids and apolipoproteins: Triglycerides and cholesterol were measured in heparin plasma by enzymatic methods using commercially available test kits (Boehringer, Mannheim, FRG). Cholesterol of the high density lipoproteins (HDL) was measured after precipitation of very low density lipoproteins (VLDL) and low density lipoproteins (LDL) with phosphotungstate-$MgCl_2$. VLDL/LDL-cholesterol and LDL-cholesterol were then calculated by difference and according to the formula of FRIEDEWALD [18], respectively. The apolipoproteins (apo) A-I and B were measured by immunonephelometric methods as described elsewhere [19,20].

Platelet function tests: Platelet aggregation was measured turbidimetrically according to BORN [21] using a two-channel aggregometer, model ELVI 840 (Elvi Logos, Milan, Italy). After calibration with PRP and PPP the aggregation was triggered by adding 20 µl solutions of PAF, ADP or collagen to 200 µl PRP at a cuvette temperature of 37°C and a constant stirring speed of 900 rpm. Platelet responses were measured as maximum increase in light transmission (ΔT) occurring within 1 and 3 min after adding the inducer. Additionally, ADP- and PAF-concentrations response curves were constructed and the concentration required to produce a half-maximum aggregation response (EC_{50}) was red by interpolation. ADP, PAF, and collagen were used in final concentrations of 0.5-15 µM, 0.02-10 µM, and 5 µg/ml, respectively.

Moreover, platelet responses were studied in whole blood . For this purpose anticoagulated blood was placed into an aggregometer cuvette and stirred for 10 min. Aliquots were taken before and after stirring, transferred into ammonium oxalate/xylocitin and counted for the number of single platelets by use of phase contrast microscopy. Spontaneous aggregation was expressed as the percentage loss of single platelets obtained after stirring.

Statistical analyses: The variables of both groups were tested for normal distribution using the KOLMOGOROV-SMIRNOV test. Statistical significances between group means were assessed by the two-tailed STUDENT's t test and in one case by the paired WILCOXON's rank test. Linear correlations were calculated to evaluate relationships between various parameters. Univariate discriminant analysis was performed by setting cut off points according to the criterion of minimal apparent error rate representing the sum of falsely positive and falsely negative classified individuals.

RESULTS

Seventy six male subjects, 36 healthy volunteers and 40 atherosclerotic patients were included in this study. The age of the controls was 53±5 years and that of the patients was 52±7 years. Broca index was 107±12% in both groups. The subjects of the control group did not take any drug for at least two weeks prior to blood sampling. Coronary and peripheral arterial diseases were excluded by physical examination as well as by electro cardiographic examination during and after exercise. The patients had survived a myocardial infarction 1-18 years before their entry into this study and were administered with aspirin (7 cases), calcium channel blockers (17 cases) and nitrovasodilators (18 cases). They did not suffer from other diseases particularly essential hypertension and diabetes mellitus and were refrained from taking ß-blockers 2 weeks prior to blood sampling.

The first step of our analysis was to characterize the distribution and location of the biochemical parameters in the two groups. All variables were distributed normally and fulfilled the necessary conditions for the application of parametric statistical procedures. There was also a considerable overlapping of most variables for patients and controls. In spite of this fact, a comparison of the mean values revealed a series of significant differences between both groups (see Table 1). Thus, the degradation of PAF was 23% higher in serum from the case group compared with the controls. Also the serum lipoprotein profile of the patients was characterized by the typical abnormalities. Concentrations of triglycerides, VLDL/LDL-cholesterol and apo B were significantly increased and those of HDL-cholesterol and apo A-I significantly lowered. The differences observed between both groups in total- and LDL-cholesterol were statistically not significant.

To evaluate the discriminatory power of the various parameters, cut-off points and apparent error rates were calculated

(see also Table 1). According to the criterion of minimal error rate, apo B, HDL-cholesterol, and the degradation of PAF were identified as the best discriminators between patients and controls. Using these variables as single parameters more than 70% of the subjects were classified correctly. Triglycerides, VLDL/LDL-cholesterol, and apo A-I which were also significantly different in both groups resulted in higher error rates compared to the former variables.

TABLE 1

PAF-Degrading Capacity (nmol/ml x min), Lipids (mmol/l) and Apolipoproteins (g/l) of Normal Subjects and Survivors of Myocardial Infarction

Parameter	Controls (N = 36)	Patients (N = 40)	COP	AER (%)	p
Degradation of PAF	31.8±8.26	39.0±7.38	33.4	29	***
Total cholesterol	6.49±1.30	6.74±0.81	6.53	34	n.s.
HDL-cholesterol	1.45±0.27	1.19±0.26	1.28	29	***
VLDL/LDL-cholesterol	5.04±1.39	5.55±0.77	5.13	32	*
LDL-cholesterol	4.34±1.27	4.55±0.78	4.36	40	n.s.
Triglycerides	1.55±0.69	2.21±1.09	1.60	34	**
Apo A-I	117±18	104±14	108	36	***
Apo B	96±29	112±15	103	25	**

Means ± S.D. are shown. N = number of subjects; COP = cut-off point; AER = apparent error rate; n.s. = not significant ($p>0.05$); *$p<0.05$; **$p<0.01$; ***$p<0.001$;

The mean PAF-degrading capacity of serum from myocardial infarction survivors was found to be significantly increased also in a comparison with that of controls who had identical serum levels of total cholesterol, VLDL/LDL-cholesterol or apo B (see Table 2).

In addition to the degradation of PAF and some lipid parameters also the platelet aggregating behaviour was studied. To eliminate variations due to the medication of patients with

TABLE 2

Degradation of PAF (nmol/ml x min) in Serum from Survivors of Myocardial Infarction and Healthy Controls Matched for Lipid Parameters

Matched Parameter	N	Controls (Mean±SD)	Patients (Mean±SD)	p
Total cholesterol	19	32.0±7.3	37.7±7.4	**
VLDL/LDL-cholesterol	15	24.5±10	37.0±6.5	**
LDL-cholesterol	18	30.0±7.4	38.6±6.7	**
HDL-cholesterol	15	33.9±8.4	36.7±6.8	n.s.
Triglycerides	20	33.2±8.5	37.8±8.0	n.s.
Apo A-I	15	32.3±11	37.7±6.2	n.s.
Apo B	11	33.1±8.0	40.4±6.4	*

Mean values were compared by the paired WILCOXON rank sum test. N - number of pairs; n.s. - not significant ($p>0.05$); *$p<0.05$; **$p<0.01$

TABLE 3

Platelet Aggregation in Plasma from Normal Subjects and Survivors of Myocardial Infarction

Parameter	Controls (N = 36)	Patients (N = 40)	Significance
ADP [ΔT_{1min} (cm)]	1.4±1.2	1.7±1.0	n.s.
ADP [EC_{50} (µmol/l)]	2.5±1.4	2.0±0.6	$p<0.05$
PAF [ΔT_{1min} (cm)]	2.4±1.7	2.7±1.6	n.s.
PAF [EC_{50} (µmol/l)]	0.45±0.4	0.34±0.21	n.s.
Collagen [ΔT_{5min} (cm)]	3.4±1.4	3.7±1.6	n.s.
Spontaneous (%)	14.4±10.7	12.2±8.5	n.s.

Means ± S.D. are shown. N - number of subjects; n.s. - not significant ($p>0.05$);

ASA, these studies were performed with the use of ASA-treated platelets. Spontaneous aggregation in whole blood as well as the ADP-, PAF-, and collagen-induced aggregation responses were measured in PRP. As shown in Table 3, a statistically significant difference was only obtained by comparing the EC_{50} values of the ADP-induced platelet aggregation.

Linear correlation analysis of the measured parameters revealed a series of significant relationships between the degradation of PAF and the lipoprotein profil (see Table 4). Thus, PAF-hydrolysis in the control group correlated positively with the concentrations of total cholesterol, VLDL/LDL-cholesterol, LDL-cholesterol, as well as apo B. There was also an inverse correlation between the level of HDL-cholesterol and the degradation of PAF. Similar but distinctly weaker relationships were found in the patients group. In contrast, there were no statistically significant correlations between any of the platelet function values and the degradation of PAF as well as any lipid parameter.

TABLE 4

Relationships Between the Degradation of PAF and Concentrations of Lipids and Apolipoproteins

Lipid parameter	Linear correlation coefficient	
	Controls (N = 36)	Patients (N = 40)
Total cholesterol	0.6652***	0.3353*
HDL-cholesterol	-0.4829**	-0.2857n.s.
VLDL/LDL-cholesterol	0.7185***	0.4487**
LDL-cholesterol	0.6891***	0.2750n.s.
Triglycerides	0.3751*	0.2531n.s.
Apo A-I	-0.2233n.s.	-0.1943n.s.
Apo B	0.4924***	0.2954n.s.

n.s. - not significant ($p>0.05$); *$p<0.05$; **$p<0.01$; ***$p<0.001$
N - number of subjects

Besides single parameters also some ratios have been calculated for each subject (see Table 5). The well established quotients total-/HDL-cholesterol and apo B/apo A-I as well as the ratios PAF-degradation/HDL-cholesterol and PAF-degradation/apo A-I were found to be significantly increased in the

patients group. Using the ratio PAF-degradation/HDL-cholesterol as discriminator, more than 73% of the subjects were classified correctly.

TABLE 5

Means, Cut-off Points and Apparent Error Rates of Several Ratios of Variables

Ratio	Controls (Mean±SD)	Patients (Mean±SD)	COP	AER (%)	p
Total cholesterol / HDL-cholesterol	4.68±1.5	5.85±1.2	4.90	22	***
Apo B / Apo A-I	0.86±0.2	1.1±0.2	0.85	25	***
Degradation of PAF / HDL-cholesterol	23.2±9.2	34.4±10	28.0	26	****
Degradation of PAF / Apo A-I	0.28±0.1	0.38±0.1	0.32	28	***

COP - cut off point; AER - apparent error rate;
***p<0.0001; *****p<0.00001

DISCUSSION

It is generally accepted that alterations in serum lipid and lipoprotein values are correlated with atherosclerotic diseases [22]. Increased serum concentrations of total cholesterol, LDL-cholesterol, high blood triglycerides, and reduced levels of HDL are considered as important risk factors for cardiovascular diseases [14]. The present study demonstrates a series of strong relationships between serum lipoproteins, degradation of PAF and the manifestation of coronary artery diseases.

Considering the suggested role of PAF in the development of atherosclerosis [23] it seems quite surprising that the serum capacity to inactivate this highly proinflammatory phospholipid is significantly increased in serum of patients suffering from coronary artery diseases. On the other hand, the degradation

of PAF is catalyzed by a specific acetylhydrolase [5] which is associated with various lipoprotein particles, in particular those containing the apo B [7,8]. In accordance with a previous study [7] a close relationship between the concentrations of lipids and apolipoproteins and the capacity to degrade PAF was found in the control group. At present we have no rational explanation for the markedly weaker relationships found in the patients group. But there is experimental evidence that the degradation of PAF depends not only on the amount of PAF-acetylhydrolase but also on its distribution between the various lipoprotein classes [10]. Therefore, it seems probable that the increased degradation of PAF in serum from myocardial infarction survivors is attributable to differences in the composition of the lipoprotein particles. Such changes might influence the incorporation of PAF and/or the distribution of PAF-acetylhydrolase which are both known to affect the degradation of PAF in serum [10]. Although there is no proof that the relationships between the degradation of PAF in serum and atherosclerosis are causative in nature there are some hints to a possible use of serum PAF-acetylhydrolase as a risk indicator of atherosclerosis.

Using the PAF-degrading capacity of serum as an univariate discriminator, it is proved to exert effects comparably in magnitude to those of the more commonly recognized factors of total cholesterol, HDL-cholesterol and the apo's A-I and B. This finding is supported also by two other studies including patients suffering from peripheral vascular diseases [11] and diabetes mellitus (unpublished results). The group means of the degradation of PAF in serum were also significantly different by comparing subgroups which were matched for plasma levels of total-cholesterol, VLDL/LDL-cholesterol or apo B. Moreover, the quotient PAF-degradation/HDL-cholesterol was identified as a good discriminator. These results point to an additional improvement for the discrimination between low and high risk groups by measuring the degradation of PAF in serum. In spite of these results a final valuation of the predictive value of PAF degradation in serum can be deduced only from a prospective clinical trial.

Although platelet hyperreactivity is considered to play an important role in atherogenesis [24] we did only find a significant difference between patients and controls by comparing the EC_{50} values of the ADP-induced platelet aggregation. Moreover, there were also no significant correlations between serum lipid concentrations and any of the platelet function values in both groups of subjects. There are conflicting reports with respect to altered platelet function associated with coronary artery diseases [25,26] as well as correlations between serum lipid levels and platelet reactivity [27,28]. The reason for the discrepant findings may be related partly to methodical differences, or differences in the subject population groups. Considering our results, however, it has to be taken into account that the platelet studies have been performed after blocking the cyclooxygenase pathway by ASA which causes an interruption of the feedback amplification in platelet activation by prostaglandin and thromboxane synthesis [29]. Additionally, the results of our study may be influenced

by platelet inhibitory effects of the calcium channel blockers and the nitrovasodilators [30] taken by some patients.

There was also no evidence for a relationship between the platelet aggregation response towards PAF and its degradation in serum, suggesting that the interaction of PAF with platelets in plasma [31] is not regulated by the PAF-degrading enzyme the PAF-acetylhydrolase.

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EXHIBIT 5

Lupus (1994) 3, 55–58

Antibodies Against Platelet-Activating Factor in Patients with Antiphospholipid Antibodies

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We have studied the specificity of antiphospholipid antibodies in 148 patients with autoimmune diseases, 120 patients with systemic lupus erythematosus and 28 with the primary antiphospholipid syndrome. In addition, 20 patients suffering from syphilis were studied. As a control group, 64 healthy volunteers were investigated. Patient and control serum samples were tested for binding to seven different phospholipid antigens by ELISA. Interestingly, 90% of the sera from syphilis patients and 6% of the autoimmune patients exhibited a significant binding to platelet-activating factor (PAF), a molecule similar to the structure of phosphatidylcholine. In addition, the IgG fraction from one of the lupus patients, which showed a high binding activity to PAF, was further affinity-purified using both liposomes and an affinity chromatography column. Preincubation of these antibodies with PAF inhibited subsequent binding to immobilized PAF. These observations might suggest a putative interaction of antiphospholipid autoantibodies with PAF '*in vivo*', which may have, in some patients, important pathophysiological consequences.

Key Words: Thrombosis Syphilis Antiphospholipid Platelet activating factor SLE

Introduction

Antiphospholipid antibodies from patients with autoimmune diseases such as systemic lupus erythematosus (SLE) or the recently described primary antiphospholipid syndrome (PAPS)[1,2] detected with immunoenzymatic methods, usually react only with negatively charged phospholipids such as cardiolipin (CL), phosphatidic acid (PA), phosphatidylinositol (PI) or phosphatidylserine (PS)[3,4]. Reactivity against zwitterionic phospholipids was reported in some patients[5]. Platelet-activating factor (PAF) is an etherphospholipid with a molecular structure very similar to phosphatidylcholine; it has many biological functions[6]. In the early 1970s, this substance was initially found to be released by basophils during IgE-induced anaphylaxis[7,8] and subsequently also in patients with SLE[9]. It was identified as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine[10,11]. It was also shown that many other types of cells could release PAF under certain situations. These include platelets, endothelial cells, neutrophils and macrophages[12,13].

The immunogenicity of PAF was first reported by Nishihira *et al.* in 1984[14]. These authors produced antibodies that reacted *in vitro* against PAF by immunization of a mouse with this phospholipid. Later, other researchers confirmed the ability of PAF to induce a specific antibody response[15–17]. An immunoassay for the measurement of PAF levels was developed by using specific antibodies[18]. More recently, the fine specificity of anti-PAF antibodies raised in immunized rabbits was studied by Cooney *et al.*[19]

Herein we report the results of our search for antibodies against PAF in patients with SLE, PAPS, syphilis and in normal blood donors. To study its binding specificities, we also affinity-purified the plasma sample with the highest anti-PAF activity in the ELISA by using two different methods: a liposome-based technique and a chromatography column coated with PAF.

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Patients and methods

Patients

One hundred and twenty patients who fulfilled the American Rheumatism Association criteria for the classification of SLE[20], 28 patients with PAPS, 40 patients with syphilis and 64 normal subject (20 blood donors and 44 healthy hospital workers) were included in this study. Plasma samples were obtained by centrifugation (3000 rpm) of citrated blood for 20 min, aliquoted and stored at –20°C until ready for use. The patient whose plasma was chosen for the anti-PAF purification was a 70-year-old woman who had suffered from SLE since 1956 and who had the following manifestations: polyarthritis, five unexplained fetal losses, thrombocytopenia, positive tests for antinuclear and anti-DNA antibodies, positive lupus anticoagulant test and IgM anticardiolipin (20 MPL).

ELISA technique for anti-PAF

The ELISA method used to determine the presence of anticardiolipin (aCL) antibodies[21] was modified to detect antibodies against PAF. Briefly, 30 µl of PAF (Sigma) (50 µg/ml) dissolved in methanol/chloroform (3:1) were added to each well in a microtiter plate and left overnight at 4°C. The next steps were similar to those in the aCL ELISA and have been described elsewhere[21]. Positive reactions were compared with those obtained with the aCL immunoassay and quantification was done using the international units (GPL and MPL) used for aCL antibodies[21]. Non-specific binding was ruled out in all positive samples by running them in empty ELISA plates.

Affinity purification of anti-PAF antibody

Purification utilizing PAF liposomes Three ml of the patient's serum with the highest activity against PAF were mixed with 5 mg/ml PAF micelles prepared as described elsewhere[22] and incubated at 4°C overnight. On the following day this mixture was centrifuged for 1 h at 15 000 rpm and the precipitate separated. This precipitate was washed with phosphate buffered saline (PBS) and the suspension centrifuged again at 15 000 rpm for 1 h. This process was repeated twice. After the third wash the precipitate was resuspended in 1.5 M sodium iodide (NaI), vortex mixed and left to stand for 15 min. An equal volume of chloroform was then added, vortex mixed and allowed to stand and centrifuged again at 3000 rpm for 10 min. The aqueous layer that contained the affinity purified antibody was separated and dialysed against PBS overnight.

Chromatography column coated with PAF PAF (10 mg) (Sigma Chemical Co., St. Louis, MO) was mixed with 50 mg cholesterol (Sigma Chemical Co.) in a glass scintillation vial and evaporated under nitrogen as described elsewhere[23]. Ethanol (1 ml) was added and the vial was capped, placed in boiling water and swirled until the lipids were dispersed. The vials were then removed and, after cooling, a 10 ml solution of 15% acrylamide, 5% BIS acrylamide (BIO RAD, Cambridge, MA) was added, followed immediately by addition of 100 µl of ammonium persulfate (140 mg/ml) and 5 µl of TEMED. The mixture was transferred to a glass test tube, covered with parafilm and aluminum foil and allowed to polymerize overnight at 4°C. The rigid white gel was removed from the tube, rinsed with distilled water and minced with a razor blade. The gel was then homogenized using a hand operated loose fitting teflon pestle. The homogenized gel was washed three times in distilled water, allowing the gel to settle for 10 min and decanting the supernatant on each occasion. The settled gel particles were then assembled into a column (125 × 20 mm) and equilibrated with eight to ten bed volumes of PBS (0.01 M phosphate/0.15 M NaCl buffer), pH 7.3. Flow rates of 50–60 ml/h were used with only moderate compaction of the relatively rigid gel particles. Elution of affinity purified immunoglobulin was performed according to the following protocol. After equilibration, 8 ml of patient plasma diluted 1:4 in PBS at the same rate until absorbance of fractions at 280 nm was <0.01 absorbance units. Then 30 ml of eluting buffer, 0.1 M phosphate/ 0.5 M NaCl buffer, pH 7.3 were applied to the column at 40–50 ml/h. The eluate was collected in 2 ml fractions and optical density readings and anti-PAF activity (ELISA) were determined. These anti-PAF antibodies were tested against negatively charged and zwitterionic phospholipids. Some fractions were freeze-dried and reconstituted with smaller volumes of distilled water as a means of concentrating them for lupus anticoagulant testing.

Inhibition studies

Inhibition studies of the affinity-purified IgM anti-PAF antibody were performed by using a previously described method[24]. In brief, known amounts of the affinity-purified anti-PAF antibody diluted in PBS were incubated at 37°C overnight with increasing concentrations of PAF that ranged from 0.125 to 1 mg/ml. Different dilutions of these mixtures were then tested in the ELISA assay against PAF.

Coagulation studies

The lupus anticoagulant (LA) activity was measured by the ability of 0.1 ml of the affinity-purified IgM anti-PAF to prolong the diluted tissue thromboplastin time when mixed with 0.1 ml of normal plasma compared with the mixture of 0.1 ml of this plasma with 0.1 ml of Tris buffer, both measured after incubation at 37°C for 5 min.

Results

Anti-PAF activities of plasma samples

Syphilis patients displayed an average binding activity higher than the mean of the normal plus five standard deviations (SD), this difference being highly significant

Table 1 Number of anti-PAF antibody-positive patients in the different groups.

	SLE (n = 120)	*PAPS (n = 28)*	*Syphilis (n = 40)*	*Controls (n = 64)*
Anti-PAF IgG	4	3	14	0
Anti-PAF IgM	5	1	12	1
Anti-PAF IgG + IgM	1	1	4	0

Samples with >5 GPAFL and/or MPAFL units were considered positives.

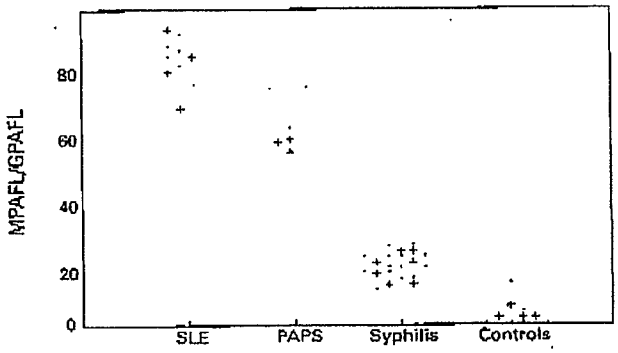

Figure 1 Distribution of anti-PAF antibody levels in the different groups of patients. MPAFL, units for IgM: GPAFL, units for IgG; SLE, systemic lupus erythematosus; PAPS, primary antiphospholipid syndrome. (•) IgM: (+) IgG.

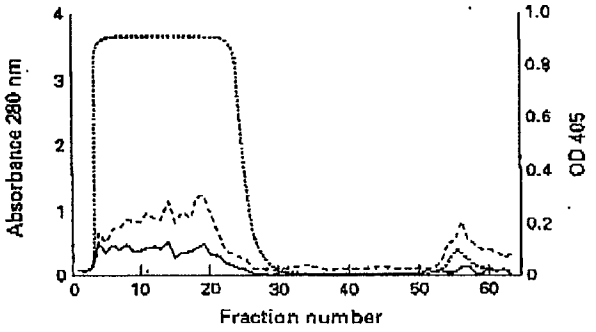

Figure 2 Column chromatography affinity-purified anti-PAF antibodies. OD, optical density. (——) aCL activity, (·····) protein concentration, (– – –) aPAF activity.

compared with the control group ($P < 0.01$). The differences between these groups and SLE and PAPS groups were not statistically significant, probably because of the heterogeneity in the latter two groups. However, 10 samples in the SLE group and five in the PAPS group showed high binding in the ELISA plates (Table I). We compared the GPL and MPL international units[23] with our optical density and created the PAF units (GPAFL and MPAFL).

In the group of normal blood donors, only one plasma demonstrated low IgM anti-PAF activity in the ELISA. Distribution of the anti-PAF antibodies levels of the four groups of patients are represented in Figure 1.

Affinity purified anti-PAF

Affinity purified anti-PAF had anti-PAF activity of 20 MPAFL. When tested by an ELISA method against negatively charged phospholipids (PS, PA, PI, CL) and against zwitterionic phospholipids (phosphatidylcholine, sphingomyelin, phosphatidylethanolamine) this showed no crossreactivity. There were no differences between liposomes and chromatography column in the affinity purified anti-PAF.

Inhibition studies

The affinity-purified IgM anti-PAF antibody when mixed with increasing concentrations of PAF was progressively inhibited. Other phospholipids such as CL or phosphatidylcholine were able to inhibit PAF binding activity, although PAF produced the highest inhibition compared with that achieved by two other phospholipids (data not shown).

Coagulation studies

Affinity-purified IgM anti-PAF antibody did not prolong the diluted tissue thromboplastin time when mixed with normal plasma compared with the mixture of this plasma with Tris buffer.

Inhibition and coagulation studies with the anti-PAF obtained with chromatography column did not differ from the liposomes affinity purified anti-PAF. The anti-PAF activity of the purified fraction was moderate (20 MPAFL) and similar to the aCL activity of the serum (Figure 2.).

Discussion

In our study, most plasma samples from patients with syphilis had low levels of antibodies that bound to PAF *in vitro* when compared with those from normal blood donors. Sera from patients with autoimmune diseases also showed significant binding. Two of these patients had high serum levels. Only differences between syphilis and normal blood donors were significant ($P < 0.01$).

Six of eight patients with autoimmune diseases (SLE and PAPS) that reached high positive values in the assay showed strong non-specific binding when tested in a plate without antigen. Only two sera showed high specific binding to PAF (IgM class). One of these patients had SLE with thrombotic manifestations and the other had autoimmune thrombocytopenic purpura. Both patients also had IgM aCL. The anti-PAF activity, the absence of crossreactivity with other phospholipids and the results of the inhibition studies performed with the affinity-purified antibody demonstrated that at least some of the antibodies against PAF may be specific and exist in some autoimmune and infectious conditions.

Although PAF binding specificities were previously reported by us and by other authors[25–27], our study was the first one that specifically studied these antibodies in various human diseases.

As most authors agree that antiphospholipid antibodies are heterogeneous, anti-PAF antibodies might represent a new specificity within this large family of autoantibodies.

A recent study showed that PAF may activate thrombolysis in response to soluble aggregates of immunoglobulin G[28]. Antibodies that block the action of PAF could then inhibit fibrinolysis and promote thrombosis[29].

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EXHIBIT 6

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A specific, sensitive radioimmunoassay for platelet-activating factor (PAF)

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A specific radioimmunoassay (RIA) has been developed for platelet-activating factor (PAF) and shown to be sensitive over the range 10–1000 pg (0.02–2 pmol). The anti-PAF antibodies showed specificity for the acetyl group at the C2 position of the PAF molecule and exhibited no significant cross-reactivity with lyso-PAF or the naturally occurring lipids including lecithin and lysolecithin. The sensitivity of the RIA was at least as good as the platelet-based assays for PAF but the RIA was simpler to perform, had a higher capacity and did not have the drawback of the inherent variability associated with the bioassays.

Key words: Platelet-activating factor radioimmunoassay; Anti-platelet-activating factor; Platelet-activating factor; Quantitation of platelet-activating factor

Introduction

Platelet-activating factor (PAF) (1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (Hanahan et al., 1980) is a potent biologically active phospholipid which induces platelet aggregation at concentrations as low as 0.1 nM. The biological actions of PAF are diverse and well-documented (Hanahan, 1986). Many cells and tissues are capable of synthesizing and releasing PAF in response to specific stimuli (Snyder, 1985; Barnes et al., 1988). Because of its potency and diverse bioactions, PAF has been implicated in many diseases including asthma, anaphylaxis, allergy, septic shock, gastrointestinal ulceration, acute graft rejection and certain kidney disorders (Braquet et al., 1987; Vargaftig and Braquet, 1987). However, unequivocal conclusions regarding the physiological role of PAF remain difficult whilst there are no precise assays available for its quantitation.

Routine and precise quantitation of low levels of PAF in large numbers of samples by standard physicochemical techniques (Hanahan and Kumar, 1987) is not a practical proposition. The most common methods used at the present time for the detection and measurement of PAF rely on the interaction of the mediator with platelets and measuring either the resultant aggregation or the release of tritiated serotonin (Hanahan and Weintraub, 1985). Although these methods are sensitive, they are not easy to perform and suffer from the inherent variability common to all bioas-

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Abbreviations: PAF, platelet activating factor (1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine); RIA, radioimmunoassay; NSS, normal sheep serum; AcT, 0.05 M sodium acetate buffer pH 6.0 containing 0.05% Tween 20 and 0.1% sodium azide; NSB, non-specific binding; lyso-PAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine.

says. Moreover, in biological samples there may be substances other than PAF that induce activation of platelets and other substances that inhibit the action of PAF on platelets. Consequently, it has been necessary to purify samples by chromatography before analysis by bioassay (Hanahan and Weintraub, 1985).

A simple and specific immunoassay for PAF should overcome most of these problems and permit both routine and accurate quantitation of this important lipid in large numbers of samples. Recently, anti-PAF antibodies with the required specificity have been produced in our laboratories (Smal et al., 1989) and have now been used in the development of simple PAF-specific radioimmunoassay (RIA) of the required sensitivity.

Materials and methods

Materials

C_{16}-PAF, C_{18}-PAF and C_{18}-dehydro-PAF were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). Polyethylene glycol MW 6000 and organic solvents were purchased from BDH Chemicals (Kilsyth, Vic., Australia) and Ajax Chemicals (Sydney, Australia) respectively. 1-palmitoyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine was a gift from Dr A. Tokumura (University of Tokushima, Japan). All other lipids were purchased from the Sigma Chemical Co. (St. Louis, MO). The IgG fraction of donkey anti-sheep antiserum was obtained from Silenus Laboratories, Melbourne, Australia. ^{125}I-labelled PAF was a kind gift from Dr J. Czarnecki, Silenus Laboratories.

Antisera

C_{12}-PAF-methylated BSA was prepared as previously described (Smal et al., 1989). Sheep received intramuscular injections of 1 mg of this antigen emulsified in Freund's complete adjuvant. 1 month and 4 months after priming, the sheep were boosted with 1 mg of antigen in Freund's incomplete adjuvant and the animals were bled 11 days after the last immunization. PAF-acetylhydrolase activity in the antiserum was destroyed by incubating serum (1 vol.) with 1 M acetic acid (1 vol.) for 6 h before adding 0.2 M phosphate buffer pH 7.2 (8 vols.). This solution was supplemented with similarly deactivated normal sheep serum (NSS).

Titre determination

Initial experiments determined the optimal dilutions of donkey anti-sheep Ig (Silenus Laboratories) and NSS required to give maximal precipitation of ^{125}I-PAF for a given level of anti-PAF. Anti-PAF antisera were titrated in 0.05 M sodium acetate pH 6.0 buffer containing 0.05% Tween 20 and 0.1% sodium azide (AcT) and supplemented with NSS to give a fixed level of sheep serum. The diluted sera were assayed as described below, and the titres taken as the dilutions of the anti-PAF anti-sera which precipitated 40–50% of the total ^{125}I-PAF.

Extraction of saliva

This was carried out according to the procedure of Bligh and Dyer (1959). Saliva (0.8 ml) was mixed with chloroform (1.0 ml) and methanol (2.0 ml) and the mixture sonicated and vortexed extensively. Water (1.0 ml) and chloroform (1.0 ml) were added and, after vortexing, the mixture was centrifuged to achieve separation of the two phases. The lower chloroform phase was evaporated and the residue reconstituted in AcT buffer (0.8 ml).

Radioimmunoassay procedure

A PAF standard solution (0.1 mg/ml in aqueous ethanol, consisting of equal parts C_{16}-PAF and C_{18}-PAF) was diluted in AcT buffer to give standard solutions over the range 0.1–25 ng/ml. Acid-treated anti-PAF antiserum was used at a dilution of 1/8000 in acid-treated NSS 1/2000 in AcT. The donkey anti-sheep Ig was diluted 1 in 250 in AcT buffer containing 6% polyethylene glycol and ^{125}I-PAF (2200 Ci/mmol, DuPont-NEN (NEK-062), Boston, MA) was added to give approx. 40,000 cpm per 100 μl. Into duplicate polystyrene RIA tubes (Disposable Products, Australia) were placed 100 μl of each of the following: sample or PAF standard solution, anti-PAF antiserum, and anti-sheep Ig/tracer. The B_0 tubes contained no PAF, and the non-specific binding (NSB) tubes contained only the anti-sheep Ig and tracer solutions, with AcT buffer added in place of sample and anti-PAF. The tubes were

incubated at room temperature for 16 h, 4 ml AcT was added and the tubes were centrifuged at 1900 $\times g$ for 25 min. After decanting the supernatants, the radioactivity remaining in the tubes was measured and the percent of tracer bound to the precipitate (% B/B_0) calculated from the formula $(B - \text{NSB})/(B_0 - \text{NSB}) \times 100$. The amount of PAF in the samples was determined from the standard curve obtained by plotting PAF concentration against $\%B/B_0$.

Inhibition studies

Solutions of some commonly occurring lipids and selected PAF analogues were formulated in AcT buffer and were then tested in the RIA, replacing the PAF standard solutions. The effects of these compounds on the assay were calculated as $\%B/B_0$.

Results

Radioimmunoassay performance

The percent of tracer bound to the antibody in the absence of PAF (B_0) ranged from 35 to 45%. However, as the labelled PAF aged, this gradually dropped to below 30%. Non-specific binding (NSB) was low, ranging from 1 to 2%.

Since natural PAF is a mixture of various alkyl analogues, with C_{16} and C_{18} analogues generally predominating, the standard chosen for the RIA was an equimolar mixture of these two analogues. Bound ^{125}I-PAF could be displaced from the antibody complex with increasing concentrations of standard PAF, generating a standard curve as shown in Fig. 1. The curve, which was linear over the range 0.5–10 ng/ml (50–1000 pg per tube), could be used to quantitate PAF from 25 pg (0.05 pmol) to 2500 pg (5 pmol) per tube. Sensitivities down to 9 pg/tube were obtained in the present studies. In four separate assays using the same batch of tracer, the values for 50% inhibition were 1.30, 1.40, 1.65 and 1.70 ng/ml. Generally, as the age of the tracer increased, these values also increased.

Specificity

The specificity of anti-PAF antibodies was determined by quantitative hapten inhibition studies

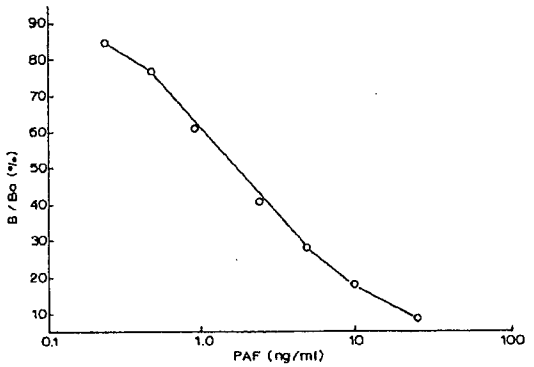

Fig. 1. A typical standard curve obtained for the PAF RIA using an equimolar mixture of C_{16}- and C_{18}-PAF together with sheep anti-PAF and ^{125}I-PAF.

using selected analogues of PAF in the RIA. The results are shown in Fig. 2. C_{16}-PAF proved to be the most reactive analogue, requiring 0.39 pmol for 50% displacement, whereas 0.48 pmol of C_{18}-dehydro-PAF and 0.72 pmol of C_{18}-PAF were required for 50% inhibition.

The acyl analogue, 1-palmitoyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (1-palmitoyl-AGPC), was poorly recognised by the antibodies and 87 pmol of this compound were required to achieve 33% inhibition.

The commonly occurring lipids were also tested for their potential to inhibit the assay (Table I) at concentrations up to 20 μg/ml. No significant inhibition was observed with any of these compounds.

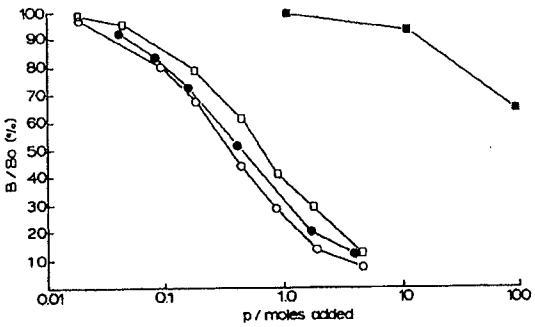

Fig. 2. Recognition of PAF analogues by sheep anti-PAF in the PAF RIA. Inhibition curves obtained with C_{16}-PAF (○), $C_{18:1}$ (dehydro)-PAF (●), C_{18}-PAF (□) and 1-palmitoyl-AGPC (■).

TABLE I

SPECIFICITY OF THE PAF RIA: EFFECTS OF COMMON LIPIDS EXAMINED FOR INHIBITORY ACTIVITY

Lipid	B/B_0 (%) at	
	20 μg/ml	4 μg/ml
Cholesteryl oleate	102	104
Triolein	101	102
Cholesterol	96	99
Oleic acid	100	100
Phosphatidyl ethanolamine	96	104
Phosphatidyl serine	103	106
Phosphatidyl inositol	106	102
Phosphatidyl choline	101	103
Sphingomyelin	99	103
Lyso-phosphatidyl ethanolamine	102	105
Lyso-phosphatidyl choline	96	103
AcT buffer only	100	100

Lyso-PAF, the primary metabolite of PAF, was tested for inhibitory potency at high and low concentrations. At less than 100 ng/ml no inhibition was observed. At very high concentrations (0.8–100 μg/ml), some displacement occurred. Cross-reactivity with the standard PAF mixture (inhibition within the range of 0.1–10 ng/ml) was 1 in 40,000.

Measurement of PAF in saliva by RIA

The PAF level in a sample of human saliva was quantitated by RIA. Lipids were extracted using chloroform-methanol-water (Bligh and Dyer, 1959) in order to eliminate any effects due to the adsorption of PAF by particulate matter in saliva (Smal, Roche, Cooney and Baldo, unpublished).

TABLE II

QUANTITATION OF PAF IN HUMAN SALIVA EXTRACT [a] BY RIA

Dilution of saliva	PAF concentration (ng/ml) [b]	
	No added PAF	With added PAF 2.5 ng/ml
1 in 2	1.6	4.7
1 in 4	0.83	3.3
1 in 8	0.35	3.0

[a] Bligh-Dyer (chloroform-methanol-water) extract.
[b] Average of duplicates

The extracts were then tested at different dilutions and with added PAF in order to determine whether the assay was correctly determining PAF levels and whether the lipids caused any interference. The PAF levels recorded were found to be within the expected range (Table II).

Discussion

In an earlier study we demonstrated that rabbit antibodies to PAF could be produced following the injection of a C_{12}-PAF analogue conjugated to methylated BSA (Smal et al., 1989). These polyclonal antibodies had the required specificity and initial attempts to develop a radioimmunoassay resulted in an assay similar to the one described here but with a sensitivity of 1 ng/ml. In an attempt to improve the sensitivity and ensure a continuity of supply of the antiserum, the PAF immunogen was injected into sheep. This resulted in the production of high titre, PAF-specific antisera suitable for RIA use.

The procedure for the present assay is straight forward, requiring only the addition of four components. The PAF-anti-PAF complex is precipitated with a second-antibody and polyethylene glycol facilitates this. Moreover, the use of the gamma emitter ^{125}I-PAF as the trace results in excellent sensitivity; alternatively ^{3}H-PAF can be used instead but this leads to diminished sensitivity (results not shown).

It has been shown that natural PAF is not a single molecular species, but a mixture of alkyl analogues, commonly C_{18}-PAF, C_{16}-PAF and C_{18}-dehydro-PAF (Mueller et al., 1984; Mallet and Cunningham, 1985; Ramesha and Pickett, 1987). Recognition of these compounds by the antibodies was similar, although not identical. The PAF standard chosen for use in the RIA was an equimolar mixture of the C_{16}- and C_{18}-PAF since this combination is probably adequate for most practical purposes.

Once the distribution in tissues and fluids of the biologically similar but structurally different PAFs has been determined, it may become necessary in the future to use a specific combination of the different analogues when measuring PAF from a specific source. It has also been demonstrated

that acyl PAF analogues may be produced concomitantly with alkyl PAF (Mueller et al., 1984; Satouchi et al., 1985; Tokumura et al., 1987) although the ratio of these two analogues is variable (ranging from 1:1 to 100:1) depending on the source. In the RIA, cross-reactivity with the acyl PAF is less than 1/500, so very little acyl PAF is likely to be detected.

The primary metabolite of PAF is lyso-PAF, which lacks the acetyl group and frequently occurs in tissues and fluids in much larger quantities than PAF (Pettipher et al., 1987; Prevost et al., 1988). The interaction of this substance with the anti-PAF antibodies is extremely weak and hence no cross-reactivity problems with lyso-PAF are envisaged. Further inhibition studies aimed at mapping the antibody combining sites in great detail have shown that there is a specific requirement for a short chain acyl group, particularly acetyl, at carbon-2 of the glycerol skeleton (Smal, Baldo and Harle, manuscript in preparation).

Since most extracts of biological samples are likely to contain large quantities of commonly occurring natural lipids, such as cholesterol, phosphatidyl choline, lyso-phosphatidyl choline etc. (see Table I), these substances were also tested in the RIA. No significant cross-reactivities were observed indicating that chromatographic purification of PAF is not necessary prior to examination in the RIA. Lipid extraction, however, is still desirable since it eliminates effects due to the non-homogeneity of biological samples and PAF-binding proteins such as albumin that may be present. Extraction is also useful in order to concentrate the analyte in cases where normal PAF levels are too low to be measured.

To test the applicability of the RIA, PAF levels in a sample of normal human saliva were quantified. Saliva was chosen since it is reported that it contains PAF (Cox et al., 1981) and is readily obtainable. When quantified by platelet aggregation following HPLC purification, PAF levels in saliva were found to be very low (for example, < 2 pg/ml) (Wardlow, 1985). By RIA, we found the PAF content to be much higher (3.2 ng/ml). Studies are now being undertaken to determine salivary PAF levels in a larger population. There appears to be no interference when measuring saliva extracts by RIA since the expected values were obtained when the sample was diluted and when extra PAF was added.

The sensitivities of the RIA and the platelet aggregation assay were found to be similar and these assays are both about ten times as sensitive as platelet degranulation procedures. The RIA offers the advantages of being reproducible and simple to perform, so that numerous samples can be processed at any one time. The platelet-based assays require a lengthy preparation time, are difficult to standardise and generally only purified PAF should be used due to the possible presence in lipid extracts of potential agonists and/or inhibitors of platelet aggregation. Accurate quantitation of PAF by this method is difficult because of the variable nature of the aggregation response. A recent improvement to platelet-based procedures has been the use of ^{3}H-PAF in radioreceptor binding assays, utilizing either whole canine platelets (Janero et al., 1988) or rabbit platelet membranes (Paulson and Nicholson, 1988). These assays are based on the displacement of tracer PAF from the PAF-receptor complex by cold PAF and offer increased reproducibility over the older methods. They do, however, suffer from the disadvantage of high non-specific binding and have the potential for detecting substances other than PAF which bind to the receptor. The sensitivities of the receptor-based assays are similar to the RIA, ranging down to 10–20 pg.

In summary, the assay described here should be applicable to a wide variety of biological samples. Since extraction (usually by the Bligh-Dyer method) is generally likely to be the only preparation required, the procedure has a high capacity. Sensitivity is likely to be sufficient for most samples and can also be increased by extraction. Using this assay it should be possible to examine rapidly large numbers of clinical samples such as blood, urine, saliva, sputum and various lavage fluids. Hence, a clearer understanding of the role of PAF in health and disease should emerge in the near future.

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